IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	§	
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MERCHAV SHOSHANA ET AL.	§	
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Serial No.: 09/890,401	§	
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Filed: 07/31/2001	§	Group Art Unit: 1651
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For: METHOD AND APPARATUS FOR MAINTENANCE AND EXPANSION	§	
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	§	Attorney Docket: 01/22310
Examiner: NAFF, DAVID M	§	

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313

DECLARATION OF SHAI MERETSKI UNDER 37 CFR 1.132

I have received my Ph.D. from the Technion Israel Institute of Technology and I am presently employed as CTO of Pluristem Life Systems Inc.

My research focuses on hemotopoietic stem cell expansion. Since the beginning of my career, I have presented my achievements at many international scientific conferences.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In this Official action, the Examiner has rejected claims 1-20 and 51-70 under U.S.C. § 103 as being unpatentable over Naughton et al. in view of Sussman et al. and Stephanopoulos et al.

The Appendix section enclosed herewith, which presents recent results obtained in our lab, illustrates that expansion of undifferentiated hematopoietic stem cells and progenitor cells requires high density stromal culture which is achieved in the presence of medium flow and not in the static cultutring conditions described in the referenced prior-art.

These results conclusively demonstrate the advantages of the present invention over prior art culturing methods, thereby providing evidence that the rejections of claims 1-20 and 51-70 under U.S.C. § 103are unfounded.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

20 March 2004

Dr. Shai Meretski
Pluristem Inc.

Enc.:
CV of Shai Meretski and Appendix

SHAI MERETZKI, Ph.D.

Personal Information

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Education

1999-2002 **Ph.D.** in Biotechnology
Technion-Israel Institute of Technology, Haifa, Israel.

Research was performed at the Haemopoiesis Unit, Rappaport Faculty of Medicine, under the supervision of Dr. Avinoam Kaduri & Prof. Dov Zipory from the Weizman Institute of Science, Rehovot, Israel and Dr. Shosh Merchav from the Technion.

Ph.D. Project: "Stationary packed bed bioreactor for propagation of transplantable human haemopoietic stem cells".

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1996-1999 **M.Sc.** The Interdepartmental Program in Biotechnology
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1981-1987 High School - The Hebrew Reali School, Haifa, Israel

Employment

2003 CTO - Pluristem Life System
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2001-2002 Biological and chemical R&D - Polyheal Ltd.
Nesher, Israel.

1997-2001 Instructor - medical students Cell Biology and Hematology
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1995-1996 The Department of Chemical Engineering – Technion
Haifa, Israel

Military Service

1987-1991 I.D.F. armed forces officer
Military rank: captain

Publications:

- Meretzki S, Kadouri A, Zipori D, Merchav S. (1998). "Maintenance and Expansion of Human Cord Blood Primitive Progenitor on Murine Stromal Cell Line." Acta Hematologica 100:20 (Abs).

- Patent: "Method and Apparatus for Maintenance and Expansion of Hemopoietic Stem Cells and/or Progenitor Cells". Publication no: WO 00/46349, August 2000.

- Meretzki S, Kadouri A, Merchav S. (2001). "Effect of Three Dimensional Stromal Cell Cultures on the Expansion of Transplantable Human Hematopoietic Stem Cells." ISHAGE 2001:41 (Abs).

- Meretzki S, Broyl A, Lapidot T, Merchav S. "Comparative Studies of the Effect of Various Stromal Cells on the Maintenance/Expansion of Human Cord Blood Stem Cells." (In press).

- Meretzki S, Kadouri A, Lapidot T, Silvian-Drachsler I, Frolov L, Merchav S. "Effect of Stroma Based Bioreactor on the Expansion of Transplantable Human Hematopoietic Stem Cells." (In press).

- Meretzki S, Kadouri A, Zipori D, Frolov L, Merchav S. (2003). "The Use of 3D Stromal Cell Cultures for the Propagation of Human Hematopoietic Stem Cells (HSC) ASH 2003:3119 (Abs).

Scholarship

1996-2002 Technion Scholarship

2001 Gutwirth Scholarship

2001 Technion Scholarship for presentation at 2001 ISAGE meeting
International Society for Hematotherapy and Graft Engineering
Quebec, Canada

1998 Gutwirth Scholarship

1998 Technion Scholarship for presentation
Molecular Biology of Hematopoiesis Convention
Bormeo, Italy

APPENDIX

The instant application suggests that growing high-density 3-D stromal culture requires a continuous flow of growth media through 3-D carriers settled within the plug-flow bioreactor. The rationale being that a flow system allows the passage of oxygen and nutrients to the cells and removal of waste materials from the cells through an active transfer rather than by diffusion.

The present invention anticipates that stromal 3-D cultures grown in a static system, similarly to the system described by Naughton and co-workers cannot reach a sufficient density to support the survival and expansion of hematopoietic stem cells and progenitor cells, which is the essence of the present invention.

The results presented hereinbelow demonstrate the superior ability of the 3-D stromal cultures, which are grown in the presence of continuous medium flow to support growth of hemoatopoietic stem cells (HSCs) and progenitor cells as compared to the static conditions described by Naughton and co-workers.

Experimental Procedures (see also pages 28-32 of the instant application)

Bioreactor - The bioreactor system is depicted in Figure 1. It containn four parallel plug flow bioreactor units [5]. Each bioreactor unit contains 1 gram of porrosive carriers (4mm in diameter) made of a non-woven fabric matrix of polyester. These carriers enable the propagation of large cell numbers in a relatively small volume. The structure and packing of the carrier have a major impact on oxygen and nutrient transfer, as well as on local concentrations and released stromal cell products (e.g., ECM proteins, cytokines). The bioreactor was maintained in an incubator of 37 °C. The flow in each bioreactor was monitored [6] and regulated by a valve [6a]. Each bioreactor contains a sampling and injection point [4], allowing the sequential seeding of stromal and haemopoietic cells. Culture medium was supplied at pH 7.0 [11] from a reservoir [1]. The reservoir was supplied by a filtered [3] gas mixture containing air/CO_2/O_2 [2] at differing proportions in order to maintain sufficient dissolved oxygen at exit from the column, depending on cell density in the bioreactor. The O_2 proportion was suited to the level of dissolved O_2 at the bioreactor exit, as was determined by a monitor [12]. The gas mixture was supplied to the reservoir via silicone tubes. The culture medium was passed through a separating container [7], which enabled collection of circulating, nonadherent cells. Circulation of the medium

was obtained by means of a peristaltic pump [9] operating at a rate of 0.1-3 ml/minute. The bioreactor units were equipped with an additional sampling point [10] and two containers [8, 11] for continuous medium exchange at a rate of 10-50 ml/day. The use of four parallel bioreactor units enables periodic dismantling for purposes such as cell removal, scanning electron microscopy, histology, immunohistochemistry, RNA extraction, etc.

The glassware was designed and manufactured at the Technion (Israel) and connected by silicone tubing (Degania, Israel). The carriers were rotated overnight in phosphate buffered saline (PBS; Beit Ha'Emek Industries, Israel) without Ca^{+2} and Mg^{+2}, followed by removal of the PBS and released debris. Each column was loaded with 10ml packed carrier. The bioreactor was filled with PBS-Ca-Mg, all outlets were sealed and the system was autoclaved (120 °C, 30 minutes). The PBS was removed via container [8] and the bioreactor was circulated in a 37°C incubator with 300 ml Dulbecco's high-glucose medium (DMEM; GIBCO BRL) containing 10% eat-inactivated fetal calf serum (FCS; Beit Ha'Emek Industries, Israel) and a Pen-Strep-Nystatin mixture (100 U/ml:100 μg/ml:1.25 μn/ml; Beit Ha'Emek), for a period of 48 hours. Circulating medium was replaced with fresh in long-term culture (LTC) medium, consisting of DMEM supplemented with 12.5 % FCS, 12.5 % horse serum (Beit Ha'Emek), 10^{-4} M β-mercaptoethanol (Merck) and 10^{-6} mol/L hydrocortwasone sodium succinate (Sigma).

Stromal cell isolation and growth– Primary human marrow stromal cultures were established from aspirated marrow of hematologically healthy donors undergoing open-heart surgery. Briefly, marrow aspirates were diluted 3-fold in Hank's Balanced Salts Solution (HBSS; GIBCO BRL) and were subject to Ficoll-Hypaque (Robbins Scientific Corp. Sunnyvale, CA) density gradient centrifugation. Marrow mononuclear cells (<1.077 gm/cm^3) were collected, washed 3 times in HBSS and re-suspended in long-term culture (LTC) medium, consisting of DMEM supplemented with 12.5% FCS, 12.5% horse serum (Beit Ha'Emek), 10^{-4} M β-mercaptoethanol (Merck) and 10^{-6} mol/L hydrocortwasone sodium succinate (Sigma). Cells were incubated in 25 ml tissue culture flasks (Corning) for 3 days at 37 °C (5% CO2) and then at 33 °C (idem) with weekly culture re-feeding. Stromal cells from individual donors were employed for each bioreactor. For 3-D and monolayer studies, primary stromal cell cultures were split by trypsinization (0.25% Trypsin and

EDTA in Puck's Saline A; Beit Ha'Emek) every 10 days, to allow sufficient stromal cell expansion.

Seeding of the stromal cells: Confluent cultures of 6-week primary marrow stromal cells were trypsinized and the cells washed 3 times in HBSS, re-suspended in LTC medium (see above), counted and seeded at $5*10^4$ cells/ml in 10 ml volumes via an injection point ([4], Figure 1) onto 10 ml carriers in the glass column of the bioreactor. Immediately following seeding, circulation was stopped for 16 hours to allow the cells to settle on the carriers. Upon settlement of the stroma cells on the carriers in the bioreactor, medium flow was re-initiated at a rate of 0.1 - 1.0 ml per minute. Stromal cell growth in the bioreactor was monitored by removal of carriers and cell enumeration by the MTT method. The Primary human marrow stromal cells were grown in 3-D culture in a plug flow bioreactor for 40 days in LTC medium and reached density of $5*10^6$ cells/ml.

Stromal-stem cell cocultures: Isolated, pooled CB CD34+ cells were seeded at equivalent numbers (about 5×10^5 CD34+ cells/sample) onto monolayers stromal cells cultures (2-D), onto 3-D stromal cells cultures that were sterilely taken from the bioreactor (static 3-D) and into the bioreactor containing equivalent densities of confluent stromal cells. In each of the above-referenced groups, CD34+ cells were seeded in LTC media (1ml LTC media/1 carrier). Upon addition of the CD34+ cells to the bioreactor, medium flow was stopped for 16 hours to enable contact with stromal cells and was re-initiated at a rate of 0.1 - 1.0 ml per minute. Fifty percent of the media was replaced weekly.

CD34+ cell seeded-stromal cell carriers were sterilely removed from the bioreactor for control studies in the absence of medium exchange. Cocultures were maintained in 1ml LTC medium/carrier without cytokines at 37 °C. Fifty percent of the medium was replaced weekly.

At various times (up to 4 weeks), nonadherent cells were collected from the static cells cultures or from the circulating culture medium within the bioreactor via a container ([8], Figure 1). Adherent cells were collected via sequential trypsinization and exposure to EDTA-based dissociation buffer (GIBCO BRL), followed by gentle pipetting of the cells. To avoid the presence of stromal cells in the resulting suspension, the cells were re-suspended in HBSS + 10 % FCS and were subjected to a 60 minutes adhesion procedure in plastic tissue culture dishes (Corning), at 37 °C.

Circulating and carrier-isolated haemopoietic cells were washed, counted and assayed separately for CD34+/38-/CXCR4+ by flow cytometry.

Flow Cytometry: Cells were incubated at 4 °C for 30 minutes with saturating concentrations of monoclonal anti-CD34+PerCP (Beckton-Dickinson), anti-CXCR4-fluorescein isothiocyanate (FITC, R&D systems) and - phycoerythrin (PE, Beckton-Dickinson) antibodies. The cells were washed twice in ice-cold PBS containing 5% heat-inactivated FCS and re-suspended for three-color flow cytometry on a FACSscan (Beckton-Dickinson).

Hematopoietic cell-growth in the presence and absence of flow –CD34+ cells were seeded into the bioreactor described in the instant application, which contained a confluent 3-D culture of 40-day old primary human marrow stroma. As a control, CD34+ Cells were also seeded onto confluent static 3-D ("carrier + stroma" bar of Figures 2a-c) or 2-D cultures ("2D stroma" bar of Figures 2a-c) of primary human stroma cells or on 3-D structures without stroma cells ("carrier" bar of Figures 2a-c). The cells were seeded in LTC medium in the absence of cytokines [LTC medium: DMEM (GIBCO BRL), 12.5% heat-inactivated FCS (Beit Ha'Emek, Israel), 12.5% horse serum (HS) (Beit Ha'Emek, Israel), Pen-Strep-Nystatin mixture (Beit Ha'Emek, Israel),10^{-4} M L-glutamine (Beit Ha'Emek, Israel),10^{-4} M mercaptoethanol (Merck), 10^{-6} M hydrocortisone sodium succinate (Sigma)].

Seven days following seeding, the cultures were trypsinized and hematopoietic stem cells and progenitors were analyzed by FACS, using the surface markers CD34, CD38 and CXCR4 [Anti-CD34 - fluorescein isothiocyanate (FITC) B&D, NJ, USA, Anti-CD34 - B&D, NJ, USA , Anti-CD38 - phycoerythrin (PE), Coulter, Florida, USA, Anti-CD45 - fluorescein isothiocyanate (FITC)B&D, NJ, USA, Anti-CXCR4-fluorescein isothiocyanate (FITC) B&D, NJ, USA]. .

Brief description of the figures

FIG. 1 illustrates the three dimensional plug flow bioreactor system. 1-medium reservoir; 2 - gas mixture container; 3 - gas filters; 4 - injection points; 5 - plug or container of plug flow bioreactor ; 6 - flow monitors; 6a - flow valves; 7 - conditioned medium collecting/separating container; 8 - container for medium exchange; 9 - peristaltic pump; 10 - sampling point; 11- container for medium exchange; 12 - 02 monitor; 14 - steering device; PH - pH probe.

FIGs. 2a-c are histograms showing the growth of CD34+ (Figure 2a), CD34+CD38- (Figure 2b) and CD34+CD38-CXCR4+ (Figure 2c) cells under the following test conditions: **Bioreactor total** - Hematopoietic cells growing in the bioreactor (3-D co-cultures + 3-D SCM); **3-D CM** – non-adherent hematopoietic cells collected from the medium circulating in the bioreactor; **Bioreactor carriers**- hematopoietic cells collected from the carriers in the bioreactor; **2-D CM** – non-adherent hematopoietic cells growing in 2-D static co-cultures; **2-D stroma** – adherent hematopoietic stem cells growing in static 2-D co-cultures; **Carrier** - hematopoietic cells growing on carriers in static cultures (without stroma); and **Carrier + stroma** - hematopoietic cells growing on 3-D static co-cultures taken from the bioreactor (similar to the system described by Naughton and co-workers.

CD34+38-CXCR4+/CD34+38-/CD34+ cells input were 115,500, 531,300, and 3,077,000 respectively.

Results are presented as the number of CD34+38-CXCR4+/ CD34+38-/ CD34+ cells taken from three independent samples in two separate experiments.

Results

Six-week old primary human marrow stromal cells were grown within the bioreactor for 40 days. Prior to seeding hematopoietic stem cells onto the stromal cells cultures, the primary human stromal cells 3-D culture originating from bone marrow was validated not including hematopoietic cells, which might influence the results of the experiment. In order to verify it, 3-D cultures of stromal cells were sterilely removed from the bioreactor and examined for the presence of hematopoietic precursors, essentially, presence of the CD34 membrane marker (data not shown).

In the next stage, confluent 3-D cultures of hematopoietic-free stroma cells were co-incubated with hematopoietic stem cells (HSCs) and the growth of HSCs under flow conditions was compared to the growth of HSCs under static cultures, by cell counting and FACS analysis of the membrane markers CD34, CD38 and CXCR4.

Noteworthy is that, following 4-24 hours most of the HSCs which were seeded on the 3-D stroma cells cultures were found to be embedded within the stroma.

As shown in Figures 2a-c, the 3-D structure could support the growth of CD34 + 38- and CD34 + 38- CXCR4+ cells better than the suspension cultures and the stroma cells 2-D cultures. Nevertheless, following 7 days of incubation only 30% of

the initially seeded CD34+ cells remained (Figure 2a), and the number of CD34 + 38- (Figure 2b) and CD34 + 38- CXCR4+ (Figure 2c) decreased to less than 5% of the seeded cell number (i.e., input).

The static 3-D primary human stroma cells cultures that were previously removed from the bioreactor and kept in petry dishes (carrier + stroma) supported the CD34+ better than the control groups containing 3-D structures (without stroma) or 2-D stroma cultures. Noteworthy is that the static 3-D cultures (similar to the system described by Naughton and co-workers, see "carrier + stroma" bar) could not support the maintenance of hemoatopoietic stem cells and progenitor cells and the remaining CD34 + 38- and CD34 + 38- CXCR4+ cells in the static cultures were only 21% and 10% (respectively) of the initial cell number, which indicates the inability of the static cultures to support HSCs.

In sharp contrast, the plug flow bioreactor, which contained the 3-D primary human stroma cells cultures, supported the growth of CD34+ cells significantly better (i.e., 3 fold, see "bioreactor total" bar vs. "carrier + stroma" bar) than the static 3-D stroma cells cultures. The 3-D culture contained within the bioreactor supported the different hematopoietic precursors more than 10 fold better than control groups containing the 2-D cultures or the 3-D carrier structure (without stroma cells). Unlike the static 3-D cultures ("carrier +stroma" bar), which did not support the CD34+ 38- cells, the bioreactor containing 3-D stroma cells cultures supported the expansion of these cells. The CD34+ 38- cells were found within the stroma cells 3-D cultures (45%) and within the growth media (55%).

Similar results were found in connection with the ability of the bioreactor system to support CD34+ 38-CXCR4+ cells (Figure 2c). While 3-D stroma cells static cultures could hardly support the maintenance of less than 10% of the seeded CD34+ 38- CXCR4+ cells, the plug-flow bioreactor system was able to support 2-fold expansion of these cells. 60 % of these HSCs were found within the circulating growth media and 40% were found within the 3-D stroma cells cultures.

Altogether, these results prove the need for a flow system bioreactor to support the 3-D stroma cells cultures to thereby allow HSCs expansion.

Fig. 1

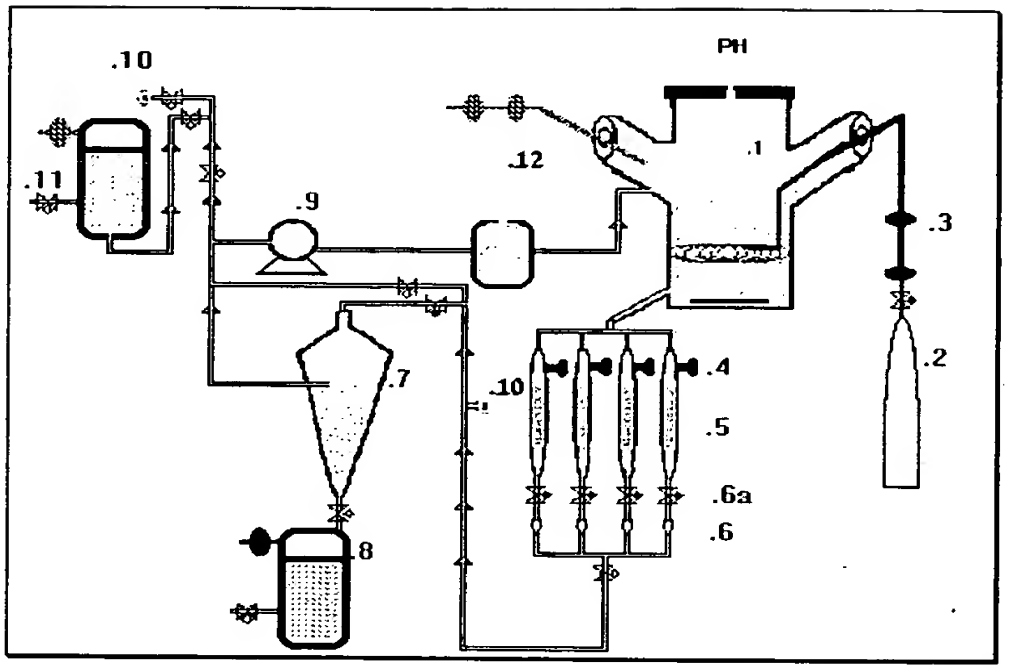

Fig. 2a

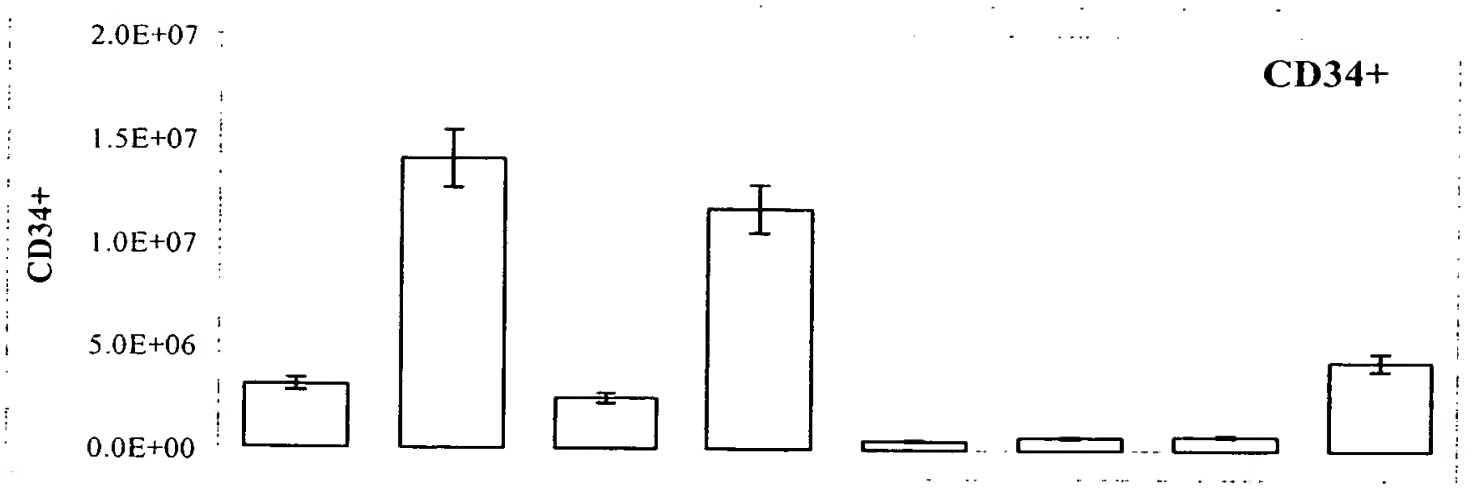

Fig. 2b

Fig. 2c